Reinvestigation of the proposed folding and self-association of the Neuropeptide Head Activator

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Abstract

The Neuropeptide Head Activator (HA), pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe (pGlu is pyroglutamic acid), is involved in head-specific growth and differentiation processes in the freshwater coelenterate *Hydra attenuata*. Peptides of identical sequence have also been isolated from higher-organism tissues such as human and bovine hypothalamus. Early studies by molecular sieve chromatography suggested that HA dimerizes with high affinity ($K_d \approx 1$ nM). This dimerization was proposed to occur via antiparallel β-sheet formation between the Lys_7-Phe_{11} segments in each HA molecule. We conducted biophysical studies on synthetic HA in order to gain insight into its structure and aggregation tendencies. We found by analytical ultracentrifugation that HA is monomeric at low millimolar concentrations. Studies by ^{1}H-NMR revealed that HA did not adopt any significant secondary structure in solution. We found no NOEs that would support the proposed dimer structure. We probed the propensity of the Lys_7-Phe_{11} fragment to form antiparallel β-sheet by designing peptides in which two such fragments are joined by a two-residue linker. These peptides were intended to form stable β-hairpin structures with cross-strand interactions that mimic those of the proposed HA dimer interface. We found that the HA-derived fragments may be induced to form intramolecular β-sheet, albeit only weakly, when linked by the highly β-hairpin-promoting D-Pro-Gly turn, but not when linked by the more flexible Gly-Gly unit. These findings suggest that the postulated mode of HA dimerization and the proposed propensity of the molecule to form discrete aggregates with high affinity are incorrect.

Keywords: Neuropeptide Head Activator; β-sheet; β-hairpin; equilibrium analytical ultracentrifugation

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The Neuropeptide Head Activator (HA), pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe (pGlu is pyroglutamic acid), was first isolated in 1981 from the freshwater coelenterate *Hydra attenuata* (Schaller and Bodenmuller 1981), where it is involved in head-specific growth and differentiation processes (for review, see Schaller and Bodenmuller 1985; Schaller et al. 1989). Identical peptides have since been purified from higher-organism tissues such as human and bovine hypothalamus, and rat intestine (Bodenmuller and Schaller 1981). Early studies revealed that the purified peptide was active only at very low concentrations, a result that was later supported by analysis of chemically synthesized HA (Birr et al. 1981; Schaller and Bodenmuller 1981). Subsequent experiments by molecular sieve chromatography suggested that the peptide forms homodimers with high affinity ($K_d \approx 1$ nM; Bodenmuller et al. 1986), which led to the hypothesis that inactivation at higher concentrations is due to self-association.

Previous attempts at conformational characterization of HA suggested that the molecule contains considerable β-sheet structure (Bodenmuller et al. 1986; Saffrich et al. 1989; Fuentes et al. 1994). This hypothesis is unusual given that small linear peptides are generally too flexible to adopt well defined conformations in aqueous solution (Wright et

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al. 1988; Creighton 1996). Bodenmuller et al. (1986) proposed that homodimerization of the peptide occurs via an antiparallel sheet-like interaction. At the proposed dimer interface, residues Lys_7 through Phe_{11} of one HA molecule are postulated to form an extended antiparallel β-sheet with the same residues of a second HA molecule (Fig. 1A). The dimerization is supposedly driven by intermolecular hydrophobic interactions between sidechains on opposing strands and by a symmetrical pair of salt bridges between Lys_7 of one HA molecule and the C-terminal carboxylate of the other.

This proposed interaction, if correct, would represent an important finding for the protein science community, because subnanomolar protein-protein interactions usually require extensive hydrophobic interactions involving burial of large surface areas between well folded protein components (Spolar and Record, Jr. 1994; Clackson and Wells 1995; Jones and Thornton 1996; Yao et al. 1998; DeLano 2002). In addition, there is growing interest in identifying peptides that can be used to bring other molecules (e.g., protein segments) together noncovalently (Arndt et al. 2000; Ghosh et al. 2000; Remy and Michnick 2001; Galarneau et al. 2002), and in development of small peptides or organic molecules that that can mimic the interfaces of protein-protein interactions (Peczuh and Hamilton 2000; Orner et al. 2001). In the present study, we reexamined the HA dimerization hypothesis via several experimental strategies.

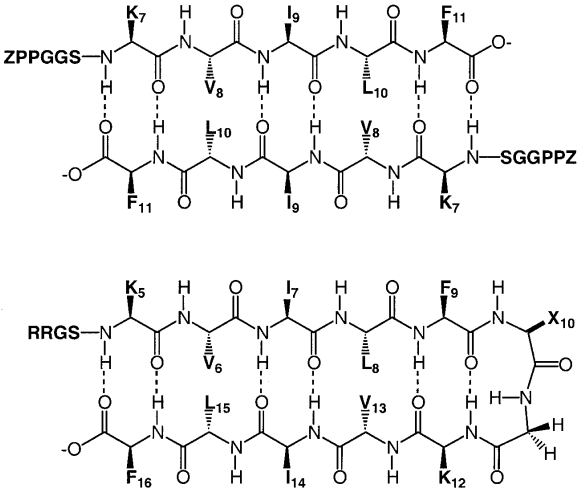

Figure 1. (*A*) Proposed HA homodimer interface (Bodenmuller et al. 1986), where Z is pyroglutamic acid. Postulated hydrogen bonds are indicated by dotted lines. The dimerization is proposed to be driven by specific contact between residues on opposing strands, and a symmetrical pair of salt-bridges between the ε-amino of Lys_7 on one HA molecule with the C-terminal carboxylate of the other HA molecule. (*B*) Hairpin conformations of peptides $(HA\text{-}f)_2$-GG and $(HA\text{-}f)_2$-pG that would mimic the interactions that are proposed to stabilize the dimer interface. Hydrogen bonding pattern between specific residues would remain in registry if residues 10 and 11 adopted a 2:2 reverse turn. In $(HA\text{-}f)_2$-GG, X_{10} = Gly, and in $(HA\text{-}f)_2$-pG, X_{10} = D-Pro.

Results and Discussion

Peptide design

Several peptides were designed and synthesized to examine the aggregational and conformational tendencies of HA-related sequences. The natural HA peptide (with the sequence ZPPGGS<u>KVILF</u>-CO_2^-, where the residues involved in the putative dimer interface are underlined) was synthesized and includes the modified glutamate residue (pyroglutamic acid, Z) at its N-terminus. The peptide HA-f (fragment) consists of the C-terminal six residues of HA (sequence $^+NH_3$-S<u>KVILF</u>-CO_2^-). This fragment has been reported to dimerize with even higher affinity than does the full-length peptide (Bodenmuller et al. 1986). In the proposed dimer interface, five of the six residues of HA-f (underlined) are involved in the antiparallel β-sheet formed between the two HA molecules. Therefore, HA-f represents the minimal sequence required for the proposed self-association.

Figure 1A shows the postulated mode of dimerization in which residues Lys_7 though Phe_{11} of one HA molecule form an antiparallel two-stranded β-sheet with the same residues of another HA molecule. We hypothesized that these interactions could be mimicked in an intramolecular fashion (Fig. 1B), and the validity of the proposed dimer interface could be tested by probing the propensity of the resulting peptide to form a β-hairpin. This configuration could be achieved by linking two segments corresponding to the C-terminal five residues of HA with a dipeptide loop. The propensity of the resulting peptides to form β-hairpins is easily evaluated using biophysical techniques and provides a strategy to detect interactions without complications due to nonspecific aggregation. Peptides $(HA\text{-}f)_2$-GG and $(HA\text{-}f)_2$-pG were designed in this fashion. Fragments of HA were linked with either a flexible Gly-Gly loop [$(HA\text{-}f)_2$-GG has the sequence $^+NH_3$-RRGS<u>KVILF</u>-GG-<u>KVILF</u>-CO_2^-], or a β-hairpin-promoting D-Pro-Gly loop [$(HA\text{-}f)_2$-pG has the sequence $^+NH_3$-RRGS<u>KVILF</u>-pG-<u>KVILF</u>-CO_2^-, where the lowercase letter denotes D absolute configuration]. An Arg-Arg-Gly-Ser segment was included at the N-termini of these peptides to improve solubility and discourage aggregation. The C-termini were left free to allow a potential electrostatic interaction between the carboxy group and the Lys_5 sidechain on the opposing strand. A similar interaction is postulated to be important for HA dimerization (Bodenmuller et al. 1986).

A β-hairpin with specific residue interactions mimicking those of the proposed interface could be envisioned if the Gly-Gly linker region and flanking residues of $(HA\text{-}f)_2$-GG adopted a 2:2 β-hairpin loop (see Fig. 1; Sibanda et al. 1989). β-Hairpin formation with this type of loop, in which the two loop residues correspond to residues *i+1* and *i+2* of

a β-turn, would maintain the registry of the hydrogen bond network of the postulated intermolecular HA dimer interface and would allow the same specific interactions that are proposed to stabilize the HA dimer. Peptide $(HA\text{-}f)_2$-pG is very similar to $(HA\text{-}f)_2$-GG, but the flexible Gly-Gly linker has been replaced by the highly hairpin-promoting D-Pro-Gly linker. The D-Pro-Gly segment strongly favors formation of type I′ and type II′ β-turns (as residues *i+1* and *i+2*). These unusual types of β-turn are commonly observed at the loops of 2:2 β-hairpins in proteins (Wilmot and Thornton 1988). The D-Pro-Gly segment has been shown to promote β-hairpin formation in several short designed peptides (Haque et al. 1996; Haque and Gellman 1997; Stanger and Gellman 1998; Das et al. 2001). Furthermore, the incorporation of D-Pro into reverse turns has also been employed in several instances to stabilize antiparallel β-sheet interactions between short strand segments derived from natural proteins outside the context of the natural tertiary structure (Struthers et al. 1996; Haque and Gellman 1997; Espinosa and Gellman 2000; Kaul et al. 2001). The Gly-Gly segment in $(HA\text{-}f)_2$-GG endows the linker with high flexibility, and any β-hairpin structure observed for $(HA\text{-}f)_2$-GG would be attributable largely to the propensity of the strand segments to form cross-strand contacts that mimic those of the postulated HA dimer interface. In contrast, the D-Pro-Gly segment of $(HA\text{-}f)_2$-pG strongly promotes formation of the β-hairpin by enforcing an appropriate reverse turn. In this case, even modest interstrand contacts should be sufficient to induce folding. The β-hairpin configurations of the designed peptides that would mimic the proposed HA dimer interface are presented in Figure 1B.

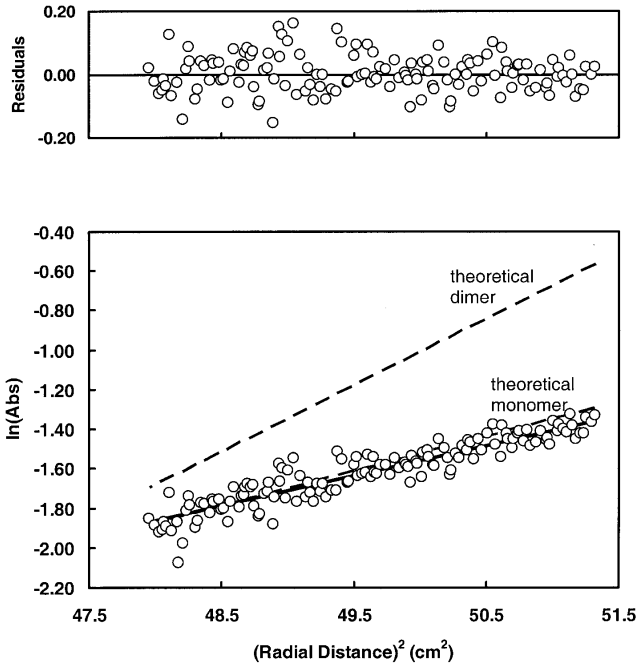

Figure 2. Representative equilibrium analytical ultracentrifugation for 1.0 mM HA in aqueous 50 mM acetic acid, pH 5.0 at 297 K. Absorbance data were acquired at 257 nm. Presence of a single species is indicated by linearity of the data. The slope is directly proportional to the molecular weight. Linear least-square regression analysis yielded molecular weight estimates consistent with HA monomer. The data shown were acquired at a rotor speed of 56 krpm, resulting in an experimental mass of ~1024 g mole^{-1} (theoretical mass of HA is 1126 g mole^{-1}). Discrepancies between observed and predicted masses may be due to nonideality effects. The fits were judged as adequate by randomness of the residuals about zero (*top*). Fit is shown as a solid line. Theoretical plots for monomer and dimer are also shown, as dashed lines.

Aggregational and conformational tendencies of HA and HA-f

Equilibrium analytical ultracentrifugation (AU) at concentrations of 1.0 mM and 1.3 mM in aqueous 50 mM acetic acid, pH 5.0, revealed that HA was predominantly monomeric under these conditions (Fig. 2). The observation that HA is monomeric at low millimolar concentrations contradicts earlier conclusions based on molecular sieve chromatography, in which HA was thought to elute predominantly as a dimer at concentrations as low as 10^{-11} M (Bodenmuller et al. 1986). In these initial studies, the dimer was thought to be stable under aqueous buffering conditions such as 0.01 M to 0.1 M Tris (pH 7.5) containing 0.1 M NaCl [the authors reported that extremely harsh conditions such as 1 M $(NH_4)SO_4$ or boiling the peptide in 0.1 M HCl were required to obtain exclusively monomeric HA]. Although our analysis of HA was performed at pH 5.0, we conclude that there are no differences in conformation or aggregation state between our sample and that previously analyzed by NMR at pH 7.2 by Saffrich et al. (1989) because the chemical shifts are identical (discussed below). From our AU studies, we observe no traces of a discrete HA dimer with high association affinity.

The peptide HA-f was reported to form a dimer with even higher affinity than HA (Bodenmuller et al. 1986). However, our analysis by AU revealed that HA-f forms large, nondiscrete aggregates at several concentrations ranging from 0.9 mM to 2.0 mM (see Electronic Supplemental Material). This behavior is not surprising based on the high content of hydrophobic and β-branched residues in HA-f. β-Branched residues have high propensity for extended β-strand conformation (Chou and Fasman 1974, 1978; Kim and Berg 1993; Minor and Kim 1994), and the hydrophobic sidechains of the Val_8, Ile_9, Leu_{10}, and Phe_{11} residues provide a driving force for self-association. Because hydrogen bond networks could be formed on either side of HA-f if the molecule were to adopt an extended conformation, the sequence may be prone to formation of infinitely large β-sheet aggregates (for examples of β-sheet self-assembly, see Lashuel et al. 2000; Powers and Kelly 2001; Powers et al. 2002). However, it is doubtful that this type of aggregation would involve specific residue-to-residue interactions as

postulated for the HA dimer interface. Nonspecific aggregation is known to occur with peptides containing a similar sequence, Lys-Leu-Val-Phe-Phe, which is believed to be involved in amyloid fibril formation (Burkoth et al. 2000; Ramirez-Alvarado et al. 2000; Cairo et al. 2002; Kelly 2002).

The ^{1}H-NMR spectra for HA were well resolved, and full TOCSY (Bax and Davis 1985) spin-system assignments were achieved using NOESY (Jeener et al. 1979) and ROESY (Bothner-By et al. 1984) experiments (complete chemical shift assignments are found in the Supplemental Material). The observed ^{1}H chemical shifts for HA were in excellent agreement with previous NMR analysis of HA (Saffrich et al. 1989). The maximum chemical shift differences were 0.01 ppm, that is, within experimental uncertainty, for all amide protons and α-protons [these types of protons are highly sensitive to secondary structure (Wishart et al. 1991, 1992)]. The similarities in chemical shifts observed for HA with previously reported values for HA suggest that there are no conformational differences between our sample and that analyzed previously in unbuffered aqueous solutions (Saffrich et al. 1989).

Chemical shifts for α-protons of residues in a β-sheet conformation are generally shifted downfield relative to α-protons of residues in the random coil state, while αH chemical shifts of residues in α-helical conformations are shifted upfield relative to random coil (Wishart et al. 1991, 1992). Three consecutive residues with consistent deviations from random coil larger than 0.1 ppm are considered evidence of secondary structure formation (Wishart et al. 1992). Figure 3A shows the deviation of observed α-proton chemical shifts from published random coil values [$\delta_{\alpha H}$(observed) − $\delta_{\alpha H}$(random coil); random coil values from Wuthrich 1986] for HA. These data suggest that HA is predominantly in a random coil state under these conditions, a finding that contradicts secondary structural predictions that HA forms 62%–67% β-sheet conformation (Bodenmuller et al. 1986; Fuentes et al. 1994).

Several NOEs were observed for HA, but none is consistent with the proposed β-sheet-like interface. Antiparallel β-sheets show several characteristic NOE patterns including crosspeaks between α-protons of residues in nonhydrogen-bonded positions on opposite strands (e.g., Val_8 αH to Leu_{10} αH in Fig. 1A), and crosspeaks between amide protons of residues hydrogen bonded to one another on opposite strands (e.g., Ile_5 NH to Ile_9 NH in Fig. 1A). None of these characteristic NOEs was observed for HA. Of the interresidue NOEs that could be unambiguously assigned, only two were between sequentially nonadjacent residues. Both of these signals were of weak intensity and between protons on Ile_9 and Phe_{11}. Two other signals may have been between sequentially nonadjacent residues but could not be unambiguously assigned due to spectral overlap (one between an Ile_9 sidechain proton and either Ser_6 NH or Leu_{10}

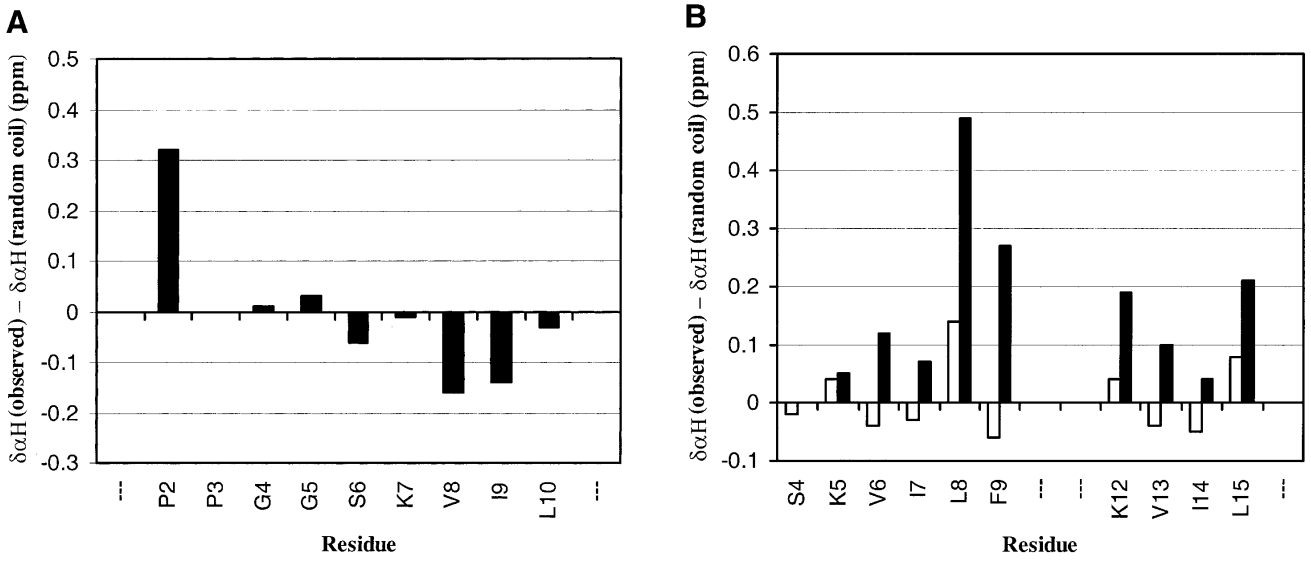

Figure 3. (*A*) Deviation of α-proton chemical shifts for HA from random coil values (Wuthrich 1986) plotted against residue. Literature random coil values for pGlu are not available, therefore this residue is not plotted. The datum for the Phe_{11} is also not plotted due to effects from the C-terminal carboxylate. The lack of consistently large deviations from random coil ($\Delta\delta\alpha H > 0.1$ ppm) implies that the peptide does not adopt significant secondary structure. Analysis was performed at 277 K in 90% H_2O/10% D_2O containing 100 mM acetic acid-d_6, pH 5.0 (uncorrected). (*B*) Chemical shift analysis for regions of $(HA\text{-}f)_2$-GG (open bars) and $(HA\text{-}f)_2$-pG (solid bars) that contain the HA-related sequences. Data acquired under the conditions described in *A*. Turn positions [Xaa_{10} and Gly_{11}, where Xaa = Gly in $(HA\text{-}f)_2$-GG and Xaa = D-Pro in $(HA\text{-}f)_2$-pG] are not plotted. Data for Phe_{16} of both peptides are also not included due to C-terminal carboxylate effects. Significant positive α-proton chemical shift deviations from random coil for $(HA\text{-}f)_2$-pG suggest some β-sheet content.

NH, and another between Phe_{11} NH and either Ser_6 NH or Leu_{10} NH). From this analysis it is not possible to distinguish inter- from intramolecular NOEs. Because we find by AU that HA is monomeric under similar conditions (NMR analysis performed at ~1 mM), it is unlikely that these NOEs are due to self-association. If these NOEs were due to intramolecular contacts, these data would suggest partial folding of the peptide to a conformation with specific interactions. However, the paucity and low intensity of the nonsequential NOEs suggests that the conformation giving rise to these NOEs is very sparsely populated. These NOE data suggest that the postulated head-to-tail mode of HA dimerization is incorrect.

Evaluation of the proposed HA dimer interface via structural analysis of (HA-f)$_2$-GG and (HA-f)$_2$-pG

Our ^{1}H-NMR analysis provides no indication of significant β-hairpin formation by (HA-f)$_2$-GG in solution. As shown in Figure 3B, no systematic deviation of α-proton chemical shifts from random coil values was observed for any region of the peptide. Furthermore, no nonsequential NOEs were observed for (HA-f)$_2$-GG. Geminal α-proton splitting for glycine residues in β-turns has been used as an indication of β-hairpin formation (Searle et al. 1999; Griffith-Jones and Searle 2000). For (HA-f)$_2$-GG, the Gly_9 protons were split very slightly (36 Hz), and chemical shifts for geminal protons of Gly_{10} were only moderately different (by 96 Hz). Vicinal $^3J_{\alpha N}$ proton couplings are also frequently used as indicators of secondary structure for proteins and peptides (Serrano 1995; Smith et al. 1996; Griffiths-Jones et al. 1998). Large values (> 8.0 Hz) are indicative of β-sheet structure, whereas smaller values (< 6.0 Hz) signify helical conformation. Most of the $^3J_{\alpha N}$ could not be determined from the proton 1D-spectrum of (HA-f)$_2$-GG due to poor dispersion; however, of the four values that could be extracted, none was above 8.0 Hz (Ser_4, 6.8 Hz; Leu_8, 7.7 Hz; Lys_{12}, 7.0 Hz; Phe_{16}, 7.9 Hz). Taken together, these data indicate that (HA-f)$_2$-GG does not adopt the β-hairpin conformation depicted in Figure 1B.

NMR evidence suggests that peptide (HA-f)$_2$-pG is at least partially folded into a β-hairpin, in contrast to (HA-f)$_2$-GG. Several of the α-proton chemical shifts for residues near the turn (Val_6–Phe_9 and Leu_{12}–Val_{13}) are suggestive of β-sheet conformation (Fig. 3B). In addition, several NOEs consistent with β-hairpin formation were observed (Fig. 4), including a long-range interaction between the α-protons of Leu_8 and Val_{13}. An additional α-proton-to-α-proton NOE was detected, but this signal could not be assigned unambiguously due to spectral overlap (Leu_{15} αH to either Val_6 αH or Ile_7 αH). Two turn-defining NH-to-NH NOEs were also observed, one between the amide protons of Phe_9 and Lys_{12}, and another between Gly_{11} and Lys_{12}. The amide ^{1}H signals were well dispersed, and the majority of the $^3J_{\alpha N}$ that could be resolved were indicative of significant β-sheet population (Lys_5, 7.2 Hz; Ile_7, 9.2 Hz; Leu_8, 9.1 Hz; Phe_9, 7.0 Hz; Lys_{12}, 8.1 Hz; Val_{13}, 7.1 Hz; Ile_{14}, 9.2 Hz; Phe_{16}, 8.0 Hz). The turn glycine geminal α-proton signals were split by 150 Hz. These data suggest that (HA-f)$_2$-pG is at least partially folded in the intended β-hairpin conformation, particularly in the turn region. This observation implies that the linked HA fragments may be induced to associate in a β-sheet mode if they are connected by a linker with a high turn propensity. However, this effect results in a β-hairpin that is only moderately stable, indicating that the cross-strand interactions are relatively weak. Based on the behavior of (HA-f)$_2$-GG and (HA-f)$_2$-pG, we conclude that the potential for interaction between the two HA-derived fragments exists, but that this interaction would not be adequate to drive dimerization at low concentration. Furthermore, we conclude that the postulated mode of HA dimerization and the strength of the aggregation as earlier reported are incorrect.

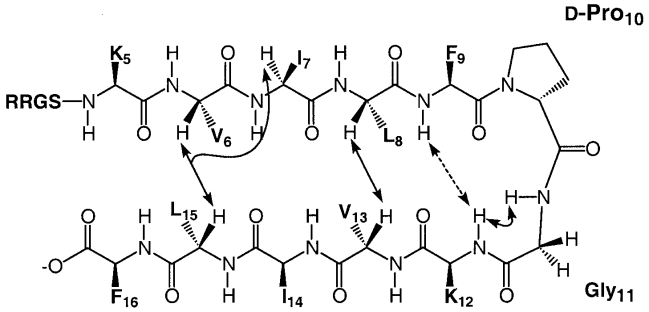

Figure 4. Long-range NOEs from 200 msec NOESY and ROESY spectra of (HA-f)$_2$-pG that are consistent with β-hairpin formation. One signal could not be unambiguously assigned due to spectral overlap (between the α-protons of Leu_{15} and Val_6 or Ile_7) and is indicated by a double-headed arrow. Extremely weak NOEs are depicted by dotted lines.

Materials and methods

Peptide synthesis and purification

All peptides were synthesized on an Applied Biosystems Model 432A Synergy automated synthesizer using standard solid-phase Fmoc chemistry with HBTU activation. The sidechain protecting groups were butoxycarbonyl (Boc) for lysine, t-butyl for serine and threonine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine, and trityl for glutamine (all amino acids were from Nova-Biochem, except for L-pyroglutamic acid, from Aldrich). The peptides were synthesized as C-terminal free acids on a methylbenzlhydrylamine (MBHA) polystyrene resin functionalized with a 4-hydroxy-methylphenoxy (HMP) linker (Applied Biosystems). Following synthesis, simultaneous cleavage from support and sidechain deprotection was achieved by treatment with a cocktail containing 85% (v/v) trifluoroacetic acid (TFA), 5% (v/v) 1,2-ethanedithiol, and 10% (v/v) thioanisole. Peptides were purified by reverse-phase HPLC (Vydac silica-C4 or -C18 semipreparative columns, 5 μm, 250 mm × 10 mm) with water/0.1% TFA and

water/acetonitrile/0.1% TFA solution mobile phases. Homogeneity of final samples was verified by analytical HPLC (Vydac silica-C4 column, 5 μm, 250 mm × 4 mm). Identity of isolated compounds was confirmed using matrix-assisted laser-desorption/ionization time-of-flight spectrometry (MALDI-TOF, measurements performed on a Bruker REFLEX II instrument).

Equilibrium analytical ultracentrifugation (AU)

Samples were prepared by dissolving the pure lyophilized peptides, isolated as the TFA salt, to the appropriate concentration in aqueous 50 mM acetic acid, pH 5.0. Equilibrium ultracentrifugation studies were conducted on a Beckman XLA ultracentrifuge at 297 K. Cells with 1.2 cm pathlengths were used, and the absorbance was monitored at 257 nm. Analysis was performed at several rotor speeds ranging from 40 to 60 krpm. Data were acquired with a 0.001 cm step size at each speed every 4 h until three consecutive spectra were identical. Linear least-squares fitting was performed in accordance with the equation $d\ (\ln c)/d\ r^2 = M(1-\nu\rho)\omega^2/2RT$, where $d\ (\ln c)/d\ r^2$ is the slope of a ln(Absorbance) versus (Radial Distance)^2 plot, ν is the partial specific volume, ρ is the solvent density, ω is the rotor speed, R is the gas constant, and T is temperature (Cantor and Schimmel 1980; Schuster and Laue 1994). Molecular weight estimates were determined from the parameter *M*. A partial specific volume of 0.786 mL g^{-1} for HA was calculated by the method of Durchschlag and Zipper (1994). A solvent density of 0.99885 g mL^{-1} was determined at 297 K using an Anton Parr DMA 5000 density meter.

Nuclear magnetic resonance (NMR)

NMR samples were prepared by dissolving the lyophilized peptides to ~1 to 2 mM in 10% D_2O/90% H_2O or pure D_2O, containing 100mM acetic acid-d_6, pH 5.0 (uncorrected). Spectra were acquired on a Varian INOVA 600 MHz spectrometer at probe temperatures ranging from 277 K to 303 K as necessary to resolve resonance overlap. A 7000 Hz spectral window was used for all acquisitions, with 80 msec, 200 msec, and 200 msec mix times for TOCSY (Bax and Davis 1985), ROESY (Bothner-By et al. 1984), and NOESY (Jeener et al. 1979) experiments, respectively. Solvent suppression in the TOCSY and ROESY spectra was achieved using a selective presaturation pulse, and in NOESY with the WET method (Ogg et al. 1994; Smallcombe et al. 1995). Typical data sets consisted of 500 to 600 free-induction decay increments of 16 to 24 transients each (corresponding to 2048 to 4096 points in f2). All spectra were processed in standard Varian software, and referenced to internal 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) standard. Although analytical ultracentrifugation was not performed on $(HA\text{-}f)_2$-GG or $(HA\text{-}f)_2$-pG, we assume that NMR studies on these peptides were conducted under nonaggregating conditions, because a 10-fold dilution of the NMR samples yielded identical one-dimensional spectra. It is unlikely that these peptides would aggregate below this concentration range (0.1 to 0.2 mM) due to their high charge density (overall charge at pH 5.0 should be +4).

Electronic supplemental material

Additional ultracentrifugation data for HA and HA-f may be found in the Supplemental Materials. Full chemical shift assignments, and a complete list of NOEs from the ^{1}H-NMR analysis of HA, are also included.

Acknowledgments

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